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TRANSLATION NO. 1754

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1754

INVESTIGATIONS ON STAPHLOCOCCAL HYALURONIDASE

III - The Effect of several "milieu factors" on Hyaluronidase Formation of Staph. aureus in vitro

Translation No. T-415-4

MAY 1966

U. S. ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK, FREDERICK, MARYLAND

INVESTIGATIONS ON STAPHLOCOCCAL HYALURONIDASE

III - The Effect of several "milieu factors" on Hyaluronidase Formation of Staph. aureus in vitro

(Following is the translation of an article by Joachim Schmidt, Institute of Medical Microbiology & Epidemology of Karl-Marx-Univ., Leipis, published in the German-language periodical, Zent. Bakt. 194 (1964) pages 59-72. Translation performed by Constance L. Lust.)

After determining experimental conditions at which optimal Staphyloccal hyaluronidase formation occurred (II paper in series), several other factors that influenced the enzyme synthesis were studied. This concerned the effects of growth factors, antibiotics and bacteriophages in order to eventually show increased hyaluronidase in the presence of hyaluronic acid, also interrelationships of enzyme formation in mixed cultures.

1.

The importance of several growth factors like thiamin (vitamin B_1), nicotinamide, biotin, riboflavin (B_2), pyridoxin (B_6) and others, for the growth of Staph. aureus has been the subject of several publications (Review by Elek 1959). However, obviously trials are lacking about the influence of those compounds that exert an effect on hyaluronidase formation. We used the synthetic medium of Gladstone (1937), which contains the amino acids cystine, leucine, valine, proline, glycine, asparagine, phenylalanine and arginine. These were present in a concentration of 30 mg % and were dissolved in a buffered salt solution containing 1% glucose. (Also see article II). The growth factors, biotin, thiamin, and nicotinamide were added, to give the following combinations. Medium #1-Basal medium; #2-basal plus biotin; #3-basal plus nicotinic acid; #4-basal plus thiamine; #5-basal plus nicotinic acid and thiamin; #6-basal plus biotin and thiamin; #7-basal plus biotin and nicotinic acid; #8-basal plus biotin, nicotinic acid and thiamin; #9-difco bouillon. The concentration of the vitamins was 1 microgram per ml. It is known from Knight (1937) that quantities of 0.2 ug per ml. of thiamin allow good growth. Biotin at 0.001 ug/ml is adequate (Porter and Pelczer 1940). High concentrations of nicotin-amide are inhibiting. Koser and Kasai (1947) found no effect at 1000 ug/ml, at 3000-5000 ug/ml growth was shown, while at 10,000 ug/ml sometimes a severe inhibition was observed.

Staph. strain M18 from a blood agar plate was grown for 20 hours at 37°C in difco bouillon, centrifuged, and the sediment was washed three times with physiological saline. After making a definite dilution (turbidity of 2000) the individual media were inoculated and incubated at 37°. After 24 and 48 hours samples were taken for hyaluronidase and turbidity determinations.

The results are presented in table 1. With medium #1 only a minimal formation of hyaluronidase was noted. Growth was also somewhat diminished compared to the control (bouillon). Addition of one of the vitamins, above all nicotinamide (#3), resulted in greater growth and greater enzyme formation. The effect on growth, of biotin and thiamin, was less pronounced. Nicotinic acid plus thiamin (#5) were best in combination. If all three growth factors were added (#8) growth and enzyme-formation were increased further, but the values were still less than those obtained with difco-bouillon. (A decrease of viscosity of 62.8% after 24 hours to 73.7% after 48 hours were determined) The differences in enzyme synthesis between medium 3,5,8 compared to #1 were larger than the range of the individual determinations.

If the values obtained for enzyme activity are compared to turbidity of #1, the differences are less than if one merely looks at the absolute hyaluronidase values. Apparently the enzyme values were somewhat greater with medium 5 (nicotinic acid plus thiamin) and 8 (all three vitamins) than those obtained with medium number 1 (no vitamins). The increased enzyme formation under the effect of vitamins is probably not a direct stimulatory effect, but is probably rather a result of increased growth. However, hyaluronidase increased more sharply during log-phase growth than the increased growth would lead one to expect. Therefore, these larger differences could be explained in this way.

2

The influence of antibiotic substances on the ability to make hyaluronidase in Staph. has not been reported. It has been reported that antibiotics usually inhibited the formations of other bacterial enzymes or even toxins.

Massart (1947) and Krampitz and Werner (1947) found an inhibition of ribonuclease in Staph. aureus. Dufrenoy and Pratt (1948) found a definite inhibition of alkaline phosphatase with low doses of penicillin, but not with high concentrations (100 IE/ml). Gillisen and Ruda (1958) could not confirm these findings. Neither in greater, nor in lower than bacteriostatic concentrations was alkaline phosphatase diminished in the presence of penicillin or neomycin. Also penicillin had no effect on acid phosphatase, although neomycin in greater than bacteriostatic concentration did exhibit some inhibitory effect. Schuler (1944) did not observe any effect on the enzymes which regulate carbohydrate metabolism. Boniece (1956) investigated the effect of antibiotics on coagulase formation in resistant strains of Staph. aureus. They measured the time required to coagulate plasma in the presence of erythromycin, tetracyclin, chloramphenicol, streptomycin and penicillin. Erythromycin was inhibitory, while tetracyclin and chloramphenicol did not. Streptomycin was slightly inhibitory at high concentration, and penicillin was not at all. Hinton and Orr (1960) reported that chloramphenicol, tetracyclin and oleandomycin in low concentrations inhibit alpha-hemolysis formation proportional to inhibition of growth. Streptomycin and bacitracin had a greater effect on inhibiting alpha-hemolysis formation than on growth.

Rosendal and Faber (1955) and Faber and Rosendal (1954) have done studies about hyaluronidase in Staph. They found that in a strain of serological group A (Type 24) the effect of penicillin was to inhibit hyaluronidase formation in the cell as well as its release from the bacterial cell. They showed that a greater production of hyaluronic acid had occurred. The inhibitory effect of penicillin on hyaluronidase formation may be because it acts on SH-groups. Dufrenoy and Pratt (1947), Pratt and Dufrenoy (1948) reported that penicillin may cause a transition of SH-groups to S-S groups. Also it is known that compounds that react with sulfhydroxyl groups inhibit enzymes (Glick and Kaufmann 1950). Faber and Rosendal also reported that in a strain of group A (Type 4) penicillin caused increased hyaluronidase production. This is not explainable by the above-mentioned mechanism.

In the present investigation we wanted to test whether hyaluronidase synthesis is altered in Staph. aureus strains in the presence of sub-bacteriostatic doses of different antibiotics. We used Staph. aureus strains E31, M55, M15, Fr48, Fr55, and 682, which showed a differential resistance to different antibiotics (penicillin, streptomycin, chloronitrin, terramycin). The strains were incubated in difco-bouillon in the presence of corresponding antibiotics, as well as controls. Samples were taken after 2, 4, 6, 8, 11, and 24 hours for turbidity and hyaluronidase measurements. The following type of experiments were used:

Staph. strain E31 plus 0.01 IE penicillin per ml; control no penicillin
" " M55 plus 0.01 IE penicillin per ml; control no penicillin
0.006 IE penicillin per ml
" " M15 plus 0.06 IE penicillin per ml; control no penicillin
0.50 IE penicillin per ml.
" " Fr48 plus 10 ug streptomycin / ml; control no antibiotic
15 ug chloronitrin / ml.
5 ug terramycin /ml.
" " Fr55 plus 10 ug streptomycin/ml; control no antibiotic
15 ug chlorontrin/ml.
" " 682 plus 10 ug streptonycin/ml; control no antibiotic
5 ug terramycin/ml.

The curves obtained for hyaluronidase for strain E31 are the same for all conditions. Growth was slower in the presence of penicillin. Strain M55 showed no difference in enzyme production in the presence of 0.01IE penicillin/ml when compared to control (Fig. 2). This was similar to what was observed with strain E31. With addition of 0.06 IE prnicillin/ml. both growth and hyaluronidase formation were markedly diminished. Strain M15 in the presence of 0.5 IE penicillin/ml showed slower growth. Enzyme formation was also slower, but reached control levels after 11 hours. Similar results were obtained with strains Fr48 and Fr55 (Fig. 3) in reference to addition of chloromitrin. Here also growth and hyaluronidase systhesis were slower. All other experiments (strain Fr55 plus streptomycin, strain Fr48 plus streptomycin, terramycin, strain 682 plus streptomycin and terramycin) showed no difference when compared to their corresponding controls without antibotics. In the presence of antibiotics, compared to controls, a decrease of hyaluronidase synthesis-later formation-occurred. This is probably not a direct effect of

the antibiotics on enzyme formation, but probably an indirect effect as a result of diminished growth. This conclusion is readily apparent when the hyaluronidase curves are compared to the growth curve.

In further studies we studied hyaluronidase formation of Staph. aureus as a result of infection by specific bacteriophages. We could find no reference in the literature about this topic.

We used Staph strain M49 and 26 in Difco-bouillon. Phage was added a) at the time bacteria were inoculated, b) 1.5 hours later after incubating at 37°C. A third set received no phages, only bacteria. Each flask contained 20 ml medium (bouillon) and the culture material was added until turbidity was visible. 0.5 ml of phage in the RTD (routine test dilution) was added. Incubation was at 37°C; samples were taken for turbidity and hyaluronidase measurements were made at 2, 3, 4, 5, 6½, 8, and 11 hours.

In figures 4 and 5 the results are summarized. With strain M49 (Fig 4) the flask with no phage added exhibited maximum enzyme formation (viscosity decrease about 65%) at the end of the logrithmic growth phase. If phage 80 was added growth was slowed markedly; after 4 hours the lytic events overcame the cells which resulted in almost completely "dissolved" the bacteria. Hyaluronidase synthesis was low (viscosity decrease 25%). It paralleled the slow growth for 4 hours and after 8 hours decreased again. No increase in free hyaluronidase occurred during the lysis period. If phage was added after 1.5 hours of incubation, then growth was initially heavier, but after 5 hours the bacteria also became lysed. Hyaluronidase reached higher values (viscosity decrease 55%) but enzyme activity only increased slightly more after 6.5 hours. After 8 hours a decrease was already apparent.

With strain 26 the same experiment proceeded differently. Little difference was seen in growth up to 5 hours in all three flasks. This probably corresponds to the duration of the log-phase of growth (Fig. 5). Lysis occurred rapidly thereafter. The curves for hyaluronidase, on the other hand, showed an identical pattern in all flasks. This may be explained in the following way: most enzyme synthesis occurrs in the log-phase of growth, and with strain 26 lysis occurred only in the last third of this growth phase.

From these studies we can conclude that in early lysis and the consequent cessetation of growth the enzyme formation is also less, if lysis occurred later then enzyme synthesis can reach control levels. Therefore lysis has no effect on the pattern of the enzyme curve. Further, the data showed that maximum hyaluronidase formation takes place in the log-phase of growth. Apparently the enzyme does not accumulate intracellularly, because in that case the enzyme activity should rise markedly after lysis of the cells.

Hyaluronidase formation in the presence of hyaluronic acid

Several bacteria showa considerable increase in hyaluronidase production in the presence of hyaluronic acid. This stimulatory effect could be demonstrated with Streptococcus (McClean 1941, Rogers 1943, Sellers 1949), Pneumococcus (McClean 1941), Clostritium perfringens (McClean and Hale 1941, Byers 1944, Rogers 1943, Hellmessen 1954) Escherichia freundii (Brunish and Mozersky 1958), but could not be found with Staphylococcus (Rogers 1945, Schwabacher 1945). The phenomenon is thought to be an adaptation to the hyaluronic acid-containing medium (Rogers 1945) and be a sign that adaptive enzyme formation was occurring.

In the present investigations we tested 5 strains of Staph. aureus, (E52, M18, M49, 971b, 1600), to see whether the presence of hyaluronic acid increased enzyme synthesis. The strains were grown in growth medium which contained 0.2% hyaluronic acid (Schering Co., Berlin), for 24 hours. Controls contained no hyaluronic acid. With strain's E52 and M18 we also looked at 6, 12 as well as 24 hours.

Growth was the same in the presence,or absence, of hyaluronic acid, and enzyme activity was not altered in any flasks. We concluded, (confirming Rogers and Schwabacher's work) that the addition of hyaluronic acid to the growth medium does not stimulate hyaluronidase formation in Staph. aureus.

Behavior of Hyaluronidase in Mixed Cultures

If two (or more) species of bacteria are grown in a growth medium, a detrimental influence may occur. This phenomenon, which may be observed in vitro as well as in vivo, is known as bacterial antagonism and has been studied extensively. This involves a complex process which seperates antagonistically-acting substances like antibiotics, certain enzymes, lipids, pigments in adaptive processes, or into a different rate of growth. According to Gillissen the influence of antagonism may also be effected through secondary factors like limiting food supply, increased metabolites, extreme pH changes and lack of oxygen. In Staph. the antagonism was studied in reference to Diphtheria-and Colibacteria and Streptococci (see Elek 1959). The opposing influences on growth have been studied most.

We found no references in the literature about the behavior of hyaluronidase formation of Staph. in mixed cultures. Thus five strains of Staph. were used (M18, 917b, E52, M49, 1600). Mixed cultures of these strains were prepared with; Pneunicoccus, Enterococcus, hemolytic Streptococcus, Neisseria catarrhalis, Diphtheria (Type Gravis), Coli, Proteus, Pyocyaneus, and Staph. epidermidis. The same concentration of bacteria suspension was inoculated, with a pipette, into medium. After 6, 12, and 24 hours incubation samples were taken for enzyme and turbidity measurements. The quantity of Staph.

in the growth culture was determined by streaking 0.1 ml of the culture on a blood agar plate. Controls of the particular strain of Staph. were always "run" for comparison; +++ (4+) was growth on the control plates. In the mixed cultures the corresponding values were ++ ,++ ,+ (3+, 2+, +) correspondingly.

The results with the strains tested were generally uniform. For example, the results obtained with Staph. strains 1600 and E52 are presented in figures 6 and 7.

In mixed cultures with Staph. epidermis, Pneunococcus, hemolytic Streptococcus and Diphtheria the same level of enzyme was found as in the control cultures of the corresponding strains of Staph. Growth also was likewise the same. Proteus, coli, and pyocyaneus gave different results in the mixed culture experiments. These germs had already largely overcome the Staphylococci after 6 hours of growth. After 24 hours the Staph. were only present in very small numbers (colony count). But, hyaluronidase formation was not suppressed much (M49, 1600 not at all; 971b, E52, M18 very slightly lowered) during this suppressed growth. Even though growth was greatly curtailed hyaluronidase was still synthesized. Apparently Staph. grew to the log-phase period, but its growth was subsequently inhibited as a result of the antagonistic effect. In mixed cultures with Neisseria catarrhalis two strains, E52 and M18, showed the same level of enzyme as controls despite inhibited growth. With strain M49 no hyaluronidase activity was measureable. The other two strains, 971b and 1600, showed growth inhibition in the presence of Neisseria catarrhalis, but still formed a small amount of enzyme. The hyaluronidase curves in the mixed cultures with Enterococci also behaved somewhat differently. Strain M18 displaced the Enterococci completely, so that the enzyme activity was exactly the same as in controls. With strain E52 and 1600 the Enterococci grew very slightly. Particularly with 1600 the enzyme activity was deminished, however.

In mixed cultures of Staph. aureus with other strains of bacteria the hyaluronidase activity is rather more variable. This is dependent on the growth intensity of Staph. aureus in the particular cultures.

It could be possible that substances that act antagonistically, like end-products of metabolism, may inhibit the formed hyaluronidase. Therefore, the following experiment was done; coli, proteus, and pyocyaneus were incubated sepeartely in medium for 24 hours. This was then steril-filtered through glass filters (Jena G5). The filtrate containing the corresponding metabolic products was added to the solution (medium) with the hyaluronidase of strains M18 and E52 and held at 4-5°C for 20 hours. No inhibition of hyaluronidase activity was noted (compared again to controls).

The low activity of hyaluronidase of Staph. aureus in mixed cultures with coli, proteus and pyocyaneus is therefore not a metabolite-inhibition, but is dependent on the antagonistic effect of these strains of bacteria. They, of course, suppress growth of the Staphylococci.

In the experiments described we studied the effects of several "milieu" factors on hyaluronidase formation. Bacterial growth factors (Nicotonic acid, biotin, thiamin) lead to increased enzyme formation indirectly as a result of increased growth. Antibiotics and phages enzyme synthesis was shown also by an indirect effect. The primary effect of antibiotics or phages was to decrease growth, antibiotics inhibited growth, phayes lysed the cells. In contrast to other bacteria Staph. aureus did not exhibit increased hyaluronidase activity in the presence of hyaluronic acid.

The results support the concept that staphylococci-hyaluronidase is a constitutive enzyme, which is always independent of the substrates in the growth medium. It is not an adaptive enzyme, which is synthesized by the microorganisms because of a need (Karstroem 1930, 1938; Leiner 1958). Karstroem called all enzymes which increase 5 times in activity because of a stimulus adaptive enzyme. The hyaluronidase of Clostridium perfringens, pneumococci, streptococci and E. freundii may be classified in this area. Adaptive stimulation did not increase hyaluronidase formation in Staph. aureus. Enzyme activity always corresponded to growth ability.

Hyaluronidase formation in mixed cultures was dependent on the microbial antagonism. The enzyme activity was present if growth of the particular strain of Staph. was not suppressed completely, but was allowed to reach its log growth phase.

Summary

The investigations on the influence of various "milieu factors" on the ability of Staph. aureus to form hyaluronidase led to the conclusion that bacterial growth substances result only indirectly through the promotion of growth in an increased formation of enzyme, while this can be reduced by antibiotics or bacteriophages, likewise in an indirect way by influencing growth. In the presence of hyaluronic acid no stimulation of hyaluronidase production occurred, and in mixed cultures it proved to be dependant on the interrelations of the bacterial antagonism.

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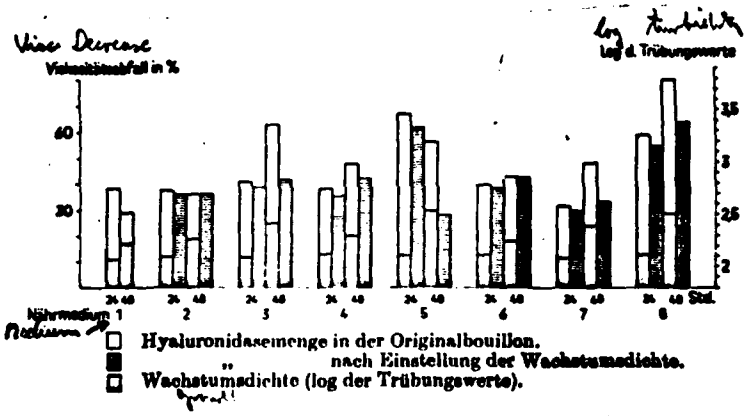


Abb. 1. Einfluß von Wuchsstoffen auf die Hyaluronidasebildungsfähigkeit des Staph. aureus.

Figure 1 - The effect of vitamins on the ability to form hyaluronidase in Staph. aureus.
(White - hyaluronidase in original medium; Hatched - hyaluronidase in medium after growth; Gray - growth (log of turbidity)

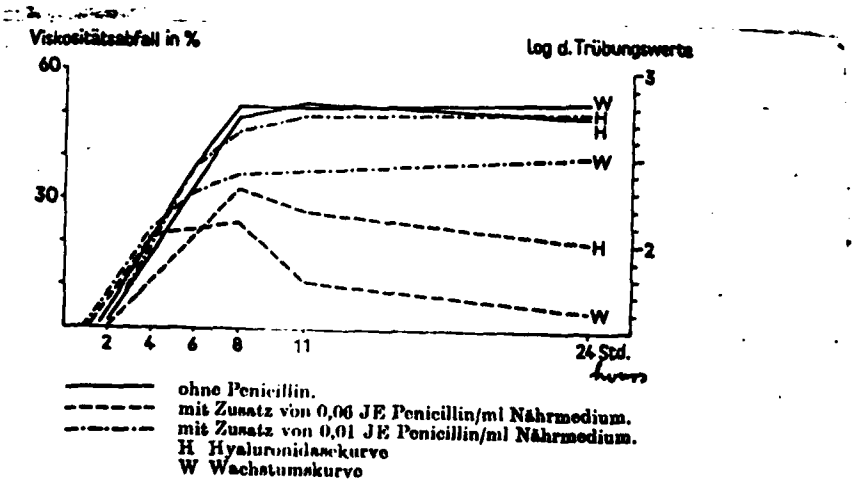


Abb. 2. Einfluß von Antibiotica auf die Hyaluronidasebildung des Staph. aureus (Staphylokokkenstamm M 55).

Figure 2 - Influence of Antibiotics on hyaluronidase formation of Staph aureus (Strain M55)
(Solid line - without penicillin; Dashed - plus 0.06JE penicillin /ml; Dot-Dash, plus 0.01 JE penicillin/ml; H hyaluronidase; W-growth

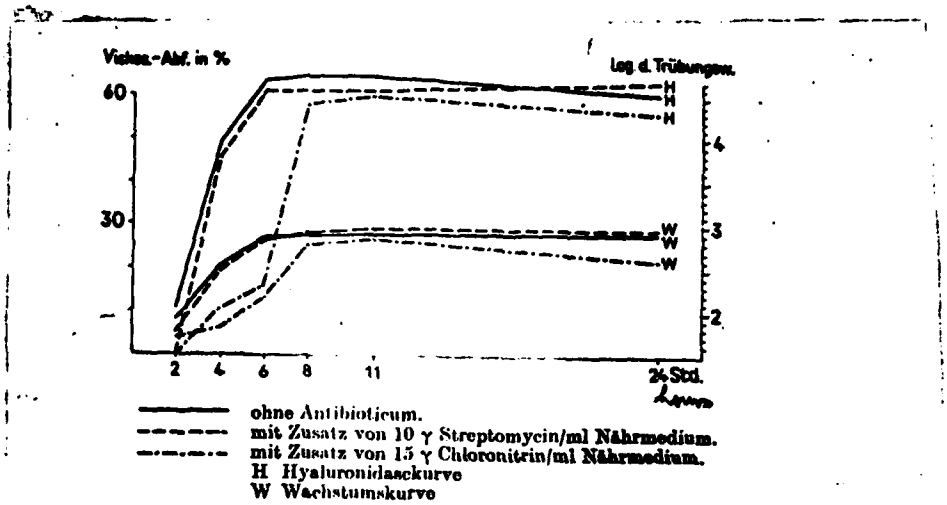


Abb. 3. Einfluß von Antibiotica auf die Hyaluronidasebildung des Staph. aureus (Staphylokokkenstamm Fr. 55).

Figure 3 - Influence of antibiotics on hyaluronidase formation of Staph. aureus (strain Fr. 55)
(Solid line, no antibiotic; Dashed, plus 10 ug streptomycen/ml.; Dot-Dash, plut 15 ug chloronitin/ml; H, hyaluronidase; W growth.

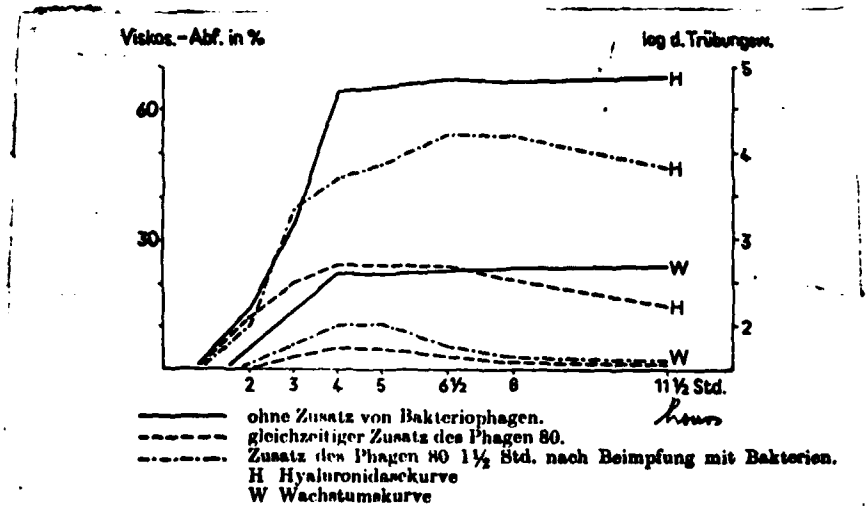


Abb. 4. Verlauf der Hyaluronidasebildung des Staph. aureus nach Zusatz von Bakteriophagen (Staphylokokkenstamm M 49).

Figure 4 - Pattern of hyaluronidase formation of Staph. aureus (Strain M49) after addition of bacteriophages
(Solid line, no phage; Dashed, plus phage 80; Dot-Dash, phage 80 added 1.5 hours later; H hyaluronidase, W growth.

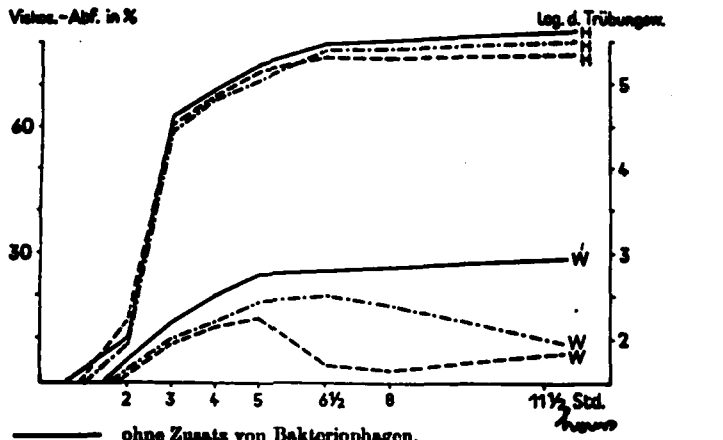


Abb. 5. Verlauf der Hyaluronidasebildung des Staph. aureus nach Zusatz von Bakteriophagen (Staphylokokkenstamm 26).

Figure 5 - Pattern of hyaluronidase formation of Staph aureus (strain 26) after adding bacteriophages.

(Solid line, no phage; Dashes, phage 75; Dot-Dash, phage 75 added 1.5 hours later; H hyaluronidase, W growth.

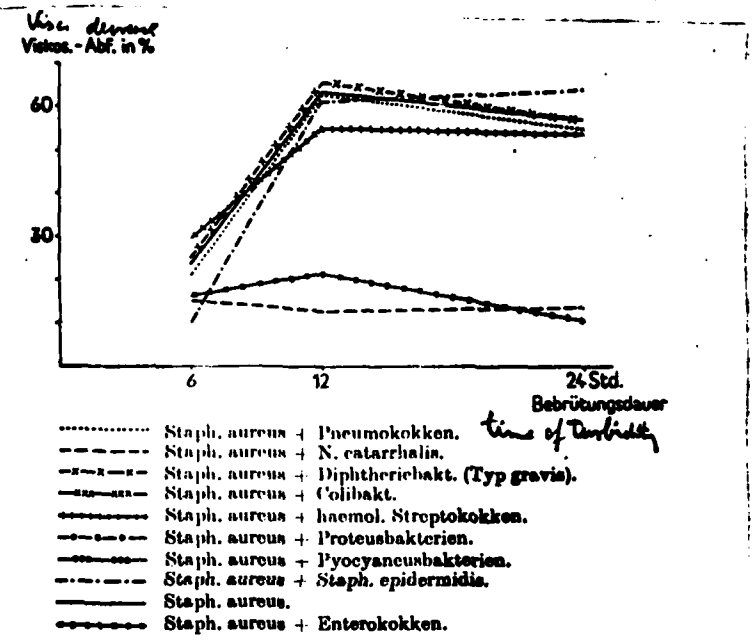
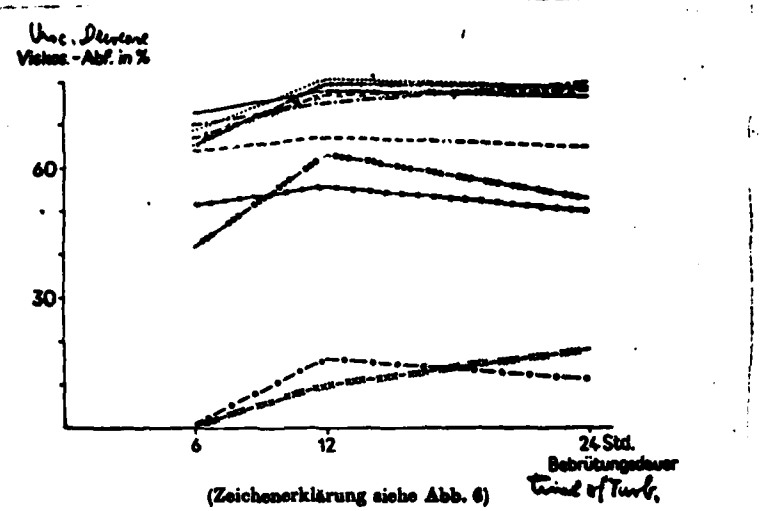


Abb. 6. Verhalten der Hyaluronidasebildung des Staph. aureus in Mischkulturen (Staphylokokkenstamm 1600).

Figure 6 - Pattern of hyaluronidase formation of Staph. aureus (strain 1600) in mixed cultures.



(Zeichenerklärung siehe Abb. 6)

Abb. 7. Verhalten der Hyaluronidasebildung des Staph. aureus in Mischkulturen (Staphylokokkenstamm E 52).

Figure 7 - Pattern of hyaluronidase formation of Staph aureus (E52) in mixed cultures.

Symbols same as for fig. 6